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(54) Title: **MAMMAL 2P DOMAIN MECHANO-SENSITIVE K⁺ CHANNEL, CLONING AND APPLICATIONS THEREOF**

(57) Abstract: The present invention relates to a protein constituting a mammalian K⁺ channel with two pore domains that produces currents whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K⁺ conditions.

Mammal 2P domain mechano-sensitive K⁺ channel,
cloning and applications thereof.

5 The present invention relates to DNA and
peptide sequence of a novel mammal member of the family of
K⁺ channel with two pore domains, called TREK2. The
invention also concerns the use of this channel in methods
for screening various compounds.

10 Potassium channel subunits containing two pore
domains form a novel class of background K⁺ channels. These
K 2P channels have unique pharmacological and functional
properties (1-10). They are active at all membrane
potentials and display very rapid kinetics of activation
15 and deactivation, and no inactivation. Their widespread
tissue distribution suggests that one of their major
physiological role is the setting of the resting membrane
potential in many different cell types. However, background
K⁺ channels with specific functional and regulatory
20 properties, as well as unique tissue distribution, have now
been cloned. These channels could be involved in more
specific functions such as epithelial K⁺ transport and
regulation of neuronal and muscular excitability (11).

25 Various K⁺ currents have been recorded *in vivo*
from neuronal, cardiac and smooth muscle cells, that form a
subfamily of background K⁺ currents sensitive to fatty
acids (12-15). Recently, fatty acid-activated K⁺ channels
have been cloned from mouse and human (2, 6, 16). These
channels named TREK1 (TWIK-Related K⁺ channel) and TRAAK
30 (TWIK-Related Arachidonic Acid-stimulated K⁺ channel)

produce quasi-instantaneous currents that are outwardly rectifying in physiological K^+ gradient. These channels have a low basal activity compared to TASK background channels (3-5). However, they can be strongly activated by application of arachidonic acid. This effect is specific of unsaturated fatty acids.

Oleate, linoleate, eicosapentaenoate and docosahexaenoate all strongly activate TREK1 and TRAAK, while saturated fatty acids such as palmitate, stearate and arachidate are ineffective (6, 17). Another efficient way for activating these channels is the application of a stretch to the cell membrane (17, 18). Both channels are activated by shear stress, cell swelling and negative pressure. They are mechano-sensitive K^+ channels. Compared to TRAAK, TREK1 has additional features.

TREK1 is inhibited by activators of protein kinases C and A (PKC, PKA). The site for PKA phosphorylation has been localized in the cytoplasmic carboxy-terminal part of the channel (17). TREK1 but not TRAAK is opened by internal acidification (19). Lowering pH_i shifts the pressure-activation relationships toward positive values and leads to channel opening at atmospheric pressure. TREK1 but not TRAAK is activated by inhalational general anesthetics, halothane and isoflurane, at concentrations used in human general anesthesia (16). Finally, TREK1 and TRAAK have different tissue distributions, the expression of TRAAK being more restricted to neuronal cells than TREK1 (2, 6, 20).

This work describes the cloning, the genomic organization, the localization and the functional

characterization of a novel human K⁺ channel with two pore domains. The molecular and functional properties of this channel indicates that it too belongs to the particular subclass of mechano-sensitive and unsaturated fatty acid-activated K⁺ channels. TREK2 is more related to TREK1 than to TRAAK, and like TREK1, it is activated by general anesthetics at clinical concentrations. Moreover, TREK2 is modulated by different types of neurotransmitter receptors. In high symmetrical K⁺ conditions, TREK2 produces currents whose current-voltage relationship is slightly inwardly rectifying.

Thus, the invention concerns a novel protein constituting a mammalian K⁺ channel with two pore domains that produces currents whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K⁺ conditions. The invention concerns more particularly a human K⁺ channel with two pore domains that produces currents whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K⁺ conditions. More particularly, the human K⁺ channel with two pore domains of the invention is constituted by or comprises the sequence of amino acids in the list of sequences under the number SEQ ID No. 2.

The invention concerns a nucleic acid molecule comprising or constituted of an encoding nucleic sequence for a mammalian K⁺ channel with two pore domains that produces currents whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K⁺ conditions or for a fragment of such a mammalian K⁺

channel. The invention also concerns a nucleic acid molecule comprising or constituted of an encoding nucleic sequence for a human K^+ channel with two pore domains that produces currents whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K^+ conditions or for a fragment of such a protein.

The invention also concerns a nucleic acid molecule which encodes for a human K^+ channel with two pore domains that produces currents whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K^+ conditions or for a fragment of this protein, whose amino acid sequence is represented in the list of sequences in the appendix under the number SEQ ID No. 2. The invention relates more particularly to a nucleic acid molecule constituted by or comprising the sequence in the list of sequences in the appendix under the number SEQ ID No. 1. Evidently the invention also concerns nucleotide sequences derived from the above sequence, for example from the degeneracy of the genetic code, and which encode for proteins presenting characteristics and properties of a mammalian K^+ channel with two pore domains whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K^+ conditions.

Another aim of the present invention is polyclonal or monoclonal antibodies directed against a mammalian K^+ channel whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K^+ conditions, a derivative or a fragment of these. These antibodies can be prepared by the methods described in the

literature. According to prior art techniques, polyclonal antibodies are formed by the injection of proteins, extracted from the epithelium or produced by genetic transformation of a host, into animals, and then recuperation of antiserums and antibodies from the antiserums for example by affinity chromatography. The monoclonal antibodies can be produced by fusing myeloma cells with spleen cells from animals previously immunised using the receptors of the invention. These antibodies are useful in the search for a novel mammalian K^+ channel with two pore domains or in the search for the human TREK2 channel homologues in other mammals.

The invention also concerns a vector comprising at least one molecule of nucleic acid above, advantageously associated with adapted control sequences, together with a production or expression process in a cellular host of a mammalian K^+ channel with two pore domains that produces currents whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K^+ conditions of the invention or a fragment thereof. The preparation of these vectors as well as the production or expression in a protein host of the invention can be carried out by molecular biology and genetic engineering techniques well known to the professional.

An encoding nucleic acid molecule for a mammalian K^+ channel with two pore domains that produces currents whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K^+ conditions or a

vector according to the invention can also be used to transform animals and establish a line of transgenic animals. The vector used is chosen in function of the host into which it is to be transferred; it can be any vector
5 such as a plasmid. Thus the invention also relates to cellular hosts expressing a mammalian K^+ channel with two pore domains whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K^+ conditions obtained in conformity with the preceding processes.

10

The invention also relates to nucleic and oligonucleotide probes prepared from the molecules of nucleic acid according to the invention. These probes, marked advantageously, are useful for hybridisation
15 detection of similar mammalian K^+ channel with two pore domains that produces currents whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K^+ conditions in other individuals or species. According to prior art techniques, these probes are put
20 into contact with a biological sample. Different hybridisation techniques can be used, such as Dot-blot hybridisation or replica hybridisation (Southern technique) or other techniques (DNA chips). Such probes constitute the tools making it possible to detect similar sequences
25 quickly in the encoding genes for mammalian K^+ channel which allow study of the presence, origin and preservation of these proteins. The oligonucleotide probes are useful for PCR experiments, for example to search for genes in other species or with a diagnostic aim.

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TREK2 is activated by general anesthetics at clinical concentrations. Consequently, this invention can also be useful in methods for identifying biologically active compounds with anesthetics properties. Thus, the invention also concerns a method for identifying a biologically active compound with anesthetics properties comprising:

(a) contacting said compound with a cellular host expressing on its surface a mammalian K^+ channel with two pore domains that produces currents whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K^+ conditions,

(b) determining the K^+ transport activity of said mammalian K^+ channel wherein an activation of K^+ transport is indicative of said compound having anesthetics properties.

This method is advantageously realised with a human K^+ channel with two pore domains that produces currents whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K^+ conditions and more preferably with a human K^+ channel constituted by or comprising the sequence of amino acids in the list of sequences under the number SEQ ID No. 2. The methods of the invention can be carried with cellular hosts which express on their surface the mammalian K^+ channel of the invention transiently or constitutively. The cellular host may be of any type which can express the protein in appropriate conformation to allow for the K^+ transport. Examples of such cellular hosts include mammalian cells, vertebrate cells and invertebrate cells.

The invention relates to pharmaceutical compositions containing a biologically active compound with anesthetics properties identified by a method described above.

5

Other advantages and characteristics of the invention will become apparent by reading the following examples concerning the cloning, genomic organization, chromosomal mapping, tissue distribution, and heterologous expression of human TREK2 and which refer to the attached drawings in which:

- Figure 1 concerns the sequence and genomic organization of TREK2. A, Alignment of TREK2, TREK1 and TRAAK channels. Relative positions of introns are indicated by arrows. M1 to M4 membrane-spanning segments and P1 and P2 pore-domains are indicated. The star shows the point of divergence between the TREK2 channel sequences from rat and human. B, Sequences at the boundaries exon-intron. Exonic sequence is in upper case. The sizes of introns is indicated. C, Dendrogram of K 2P channels cloned in human established with ClustalW and Treeview.

- Figure 2 concerns the expression of TREK2 in adult human. A, Tissue distribution analysis by RT-PCR. The amplified products were analyzed by Southern blot using specific internal primers as probes. To check the integrity of cDNAs, a GAPDH fragment was amplified. sk. muscle, skeletal muscle; sm intestine, small intestine; PBL, Peripheral Blood Leukocytes. B, Localization in the brain by Northern blot analysis. Blots were hybridized at high

stringency with specific probes. Each lane contains 2 μ g of poly(A) + RNA.

- Figure 3 concerns the biophysical properties of TREK2 currents. A, whole-cell configuration. Superimposed current traces elicited by voltage steps from -150 mV to +70 mV by increments of 20 mV. B, current-voltage (I-V) relationships in physiological (5 mM K^+) and symmetrical (150 mM K^+) K^+ gradients (800-ms voltage ramps from -130 mV to +100 mV from a holding potential of -80 mV). C, steady-state single-channel activities at the indicated potentials. Outside-out patch mode in physiological (left traces) and symmetrical (right traces) K^+ conditions. D, single-channel I-V curves of TREK2 obtained from outside-out patches in physiological (filled square) and symmetrical (open circle) K^+ conditions. Mean of five examined patches. Single-channel conductance was 128 pS at -40 mV and 100 pS at +40 mV when measured in symmetrical K^+ conditions.

- Figure 4 concerns the activation of TREK2 by a stretch of the membrane and by internal acidosis. A, reversible activation of TREK2 by membrane stretch in an inside-out patch exhibiting a low basal activity at +50 mV. B, effects of increasing stretch stimulation (in mmHg) on TREK2 activation in an multi-channel inside-out patch held at 0 mV. C, multi-channel inside-out patch. Effects of membrane voltage (as indicated) on TREK2 activation by the same membrane stretch (-75 mmHg). D, reversible activation of TREK2 by internal acidosis (pHi 5.6) in a patch displaying a low basal activity. The maximum TREK2 activation is obtained in depolarized conditions. E, multi-

channel inside-out patch. Voltage-dependence of activation by internal acidosis at pH 5.6. In A and B, the control value of pH_i was kept at 7.3.

- Figure 5 concerns the activation of TREK2 by
5 volatil anesthetics and riluzole. A, halothane (1 mM) stimulates TREK2 channel activity elicited in the whole-cell configuration. The I-V curves were obtained with a voltage-ramp protocol of 800 ms duration starting from a holding potential of -80 mV. B, activation of TREK-2
10 channel activity by chloroform ($CHCl_3$), isoflurane (Iso) and halothane. Number of cells in each experimental condition is indicated above bar. C, transient activation of TREK2 by riluzole. Evolution of the current under control conditions (1), after a 20 s application of
15 riluzole (2), after a 3 min application of riluzole (3) and after a 1 min wash (4). The voltage-clamp protocol consists of the same ramp as in A applied every 10 s. The current was monitored at +100mV. D, corresponding I-V curves of the experiment shown in C.

20 - Figure 6 concerns the activation of TREK2 by polyunsaturated fatty acids and lysophosphatidylcholine and inhibition by cAMP. A, activation by arachidonic acid (AA) of the whole-cell TREK2 current. The I-V curves were obtained with a voltage-ramp protocol of 800 ms duration
25 starting from a holding potential of -80 mV. B, activation by lysophosphatidylcholine (LPC). Same protocol as in A. C, effect of fatty acids on TREK2. AA, arachidonic acid, DHA, docosahexaenoic acid, LA, linoleic acid, PA, palmitic acid, LPC, lysophosphatidylcholine. D, regulation of TREK2
30 channel activity by cAMP. Inhibition of the current after

external application of 500 μ M CPT-cAMP. Same voltage protocol as in A.

- Figure 7 concerns the regulation of TREK2 by G-protein-coupled receptors. Left, evolution of the whole-cell TREK2 current under control conditions (1), at the steady-state effect after receptor activation (2) and after wash (3). The voltage-clamp protocol consists of a voltage-ramp of 800 ms duration starting from a holding potential of -80 mV applied every 10 s. The current was monitored at +100mV. Right, corresponding I-V curves of the experiments shown at left. The TREK2 channel was co-expressed together with 5HT4sR (A), mGluR2 (B) or mGluR1 (C) receptors. The receptors were activated by application of 5-hydroxytryptamine (5-HT) for 5HT4sR and glutamate for mGluR1 and 2. No effect on TREK2 current were seen after 5-HT and glutamate applications on COS cells transfected with only TREK2.

I. Experimental procedures.

20 I.1. Cloning of TREK2.

Sequences of two P domain K⁺ channels were used to search homologs in public DNA databases by using the tBlastn alignment program and TREK1 as the query sequence (21). This led to the identification of a genomic sequence (EMBL accession number AL133279.1) which showed significant similarities with TREK1. In order to characterize the corresponding full-length cDNA, 5'- and 3'- rapid amplifications of cDNA ends (RACE PCR) were performed on adult human brain cDNAs ligated with adaptors (22). Two antisense primers for 5'-RACE (5'-

ACTGCCGAGGTCCCAGTGGCTGCTGTT-3' [SEQ ID No. 3 in the list of sequences in the appendix] and 5'-TCTGGCTGCTCTCAAAGGGCTGCT-3' [SEQ ID No. 4 in the list of sequences in the appendix]) and two sense primers for 3'-RACE (5'-
5 GACGATCCCTGCTGTCATCTT-3' [SEQ ID No. 5 in the list of sequences in the appendix] and 5'-TTGCAGCTGTCCTCAGTAGATCG-3' [SEQ ID No. 6 in the list of sequences in the appendix]) were derived from genomic sequences.

Two successive RACE reactions were performed by
10 using anchor primers 5'-TAGAATCGAGGTCGACGGTATC-3' [SEQ ID No. 7 in the list of sequences in the appendix] and 5'-GATTTAGGTGACACTATAGAATCGA-3' [SEQ ID No. 8 in the list of sequences in the appendix]. The amplified products were subcloned into pGEMt easy (Promega) and eight clones of
15 each products were sequenced (Applied Biosystems model 373A). The entire coding sequence was amplified from human brain cDNA by PCR using a low-error rate DNA polymerase and then subcloned into the pIRES-CD8 vector to give pIRES-CD8.TREK2. Inserts from two different independent PCR-
20 ligation experiments were sequenced on both strands and found to be identical.

I.2. Analysis of TREK1, TREK2 and TRAAK distributions.

25 For RT-PCR experiment, Multiple Tissue cDNA panels were used as template according to the manufacturer's protocol (Clontech). Primers were: TREK2, sense primer 5'-CAGCCCTTTGAGAGCAGCC-3' [SEQ ID No. 9 in the list of sequences in the appendix], antisense primer 5'-
30 AAGATGACAGCAGGGATCGTC-3' [SEQ ID No. 10 in the list of

sequences in the appendix], TRAAK, 5'-GAGGCCCCGGCCAGGGGATCCTG-3' [SEQ ID No. 11 in the list of sequences in the appendix] and 5'-CTCAGTGCTCACCACCATCG-3' [SEQ ID No. 12 in the list of sequences in the appendix],
5 and TREK1, 5'-GGATTTGGAAACATCTCACCACGCACA-3' [SEQ ID No. 13 in the list of sequences in the appendix] and 5'-GATCCACCTGCAACGTAGTC-3' [SEQ ID No. 14 in the list of sequences in the appendix]. PCR conditions were 32 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. PCR
10 products were separated by electrophoresis, transferred onto nylon membranes, and probed with 32 P-labeled primers (TREK2, 5'-ACTGCCGAGGTCCCAGTGGCTGCTGTT-3' [SEQ ID No. 15 in the list of sequences in the appendix]; TRAAK, 5'-TCAGGCTGCCAGCTGGACTG-3' [SEQ ID No. 16 in the list of sequences in
15 the appendix]; TREK1, 5'-TAGCTGATCTCCAACCTCCAGCCAAG-3' [SEQ ID No. 17 in the list of sequences in the appendix]). For Northern blot analysis, multiple tissue Northern blots from Clontech were probed with the 32 P-labeled insert of pIRES-CD8. TREK2 in Ultrahyb hybridization buffer (Ambion) at
20 50°C for 18 h then washed stepwise at 55°C to a final stringency of 0.2xSSC, 0.3% SDS. Blots were then dehybridized according the manufacturer's protocol and reprobed with TREK1 and TRAAK following the same procedure. A 0.7 kb BamHI fragment from pCD8.hTREK1 and the insert
25 from pIRES-CD8.hTRAAK were used as probes. Autoradiograms were exposed 24 h at -70°C on BioMax films by using a Transcreen-HE Intensifying Screen (Kodak).

I.3. Electrophysiology in transfected COS cells.

COS cells were seeded at a density of 20,000 cells per 35 mm dish, 24 h prior transfection. Cells were transiently transfected by the classical DEAE-dextran method with 0.2 µg of pIRES-CD8. TREK2 with or without mGluR1, mGluR2 or 5HT4sR expression vectors (a generous gift of Drs JP Pin and A Dumuis, Montpellier, France).

Transfected cells were visualized 48 h after transfection using the anti-CD8 antibody-coated beads method. For whole-cell experiments, the patch electrode solution (INT) contained 150 mM KCl, 3 mM MgCl₂, 5 mM EGTA and 10 mM HEPES, adjusted to pH 7.3 with KOH; the external solution (EXT) contained 150 mM NaCl, 5 mM KCl, 3 mM MgCl₂, 1 mM CaCl₂ and 10 mM HEPES, adjusted to pH 7.4 with NaOH. For outside-out patch recordings, the pipette solution was the INT solution and the external solution was either the EXT solution (5 mM K⁺) or a K⁺-rich EXT solution which contained 150 mM KCl instead of 150 mM NaCl.

For inside-out patch recordings, pipettes were filled with the EXT solution and the bathing solution was the INT solution buffered either at pH 7.3 or at pH 5.6 in the internal acidosis experiments. Cells were continuously superfused with a microperfusion system during the experiment (0.2 ml per min) done at room temperature. A RK400 patch-clamp amplifier was used for whole-cell and single-channel recordings (Bio-Logic, Claix, France). Single-channel data were low-pass filtered at 5 KHz and digitized at 50 KHz using a DAT recorder (Bio-Logic, Claix, France). pClamp software was used to analyze whole-cell

data and Biopatch software (Bio-Logic) to analyze single-channel data.

Concentrations of volatile anesthetics were adjusted from saturated solutions (isoflurane, 15.3 mM; halothane, 17.5 mM and chloroform, 66.6 mM) in saline at room temperature (16). Mechanical stimulation was applied through an open loop pressure generating system and monitored at the level of the patch pipette by a calibrated pressure sensor (17).

10

II. Results.

II.1. Molecular cloning of TREK2.

DNA sequences produced in the frame of the human genome sequencing program are rapidly accumulating in the public high-throughput-genomic-sequences (HTGS) database. Searches of this database using the Blast sequence alignment program (21) led to the identification of human sequences restrained to a single genomic contig. The analysis of these sequences suggested the presence of introns and exons forming a gene coding for a novel K 2P subunit. Oligonucleotides were deduced from the potential exon sequences and used to clone cDNA fragments from human brain by using Rapid Amplification of CDNA Ends (RACE)-PCR. The sequence deduced from these cDNAs is 2730 bp long and contains an open reading frame (ORF) of 1617 nucleotides predicting a 538 amino acids polypeptide (Fig. 1A). This protein has the same overall structure than the previously cloned K 2P subunits. It displays four membrane spanning segments (M1 to M4), two P domains (P1 and P2) and an extended loop between M1 and P1. The dendrogram shown in

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Fig. 1C clearly indicates that this subunit is more related to TREK1 and TRAAK than to other K 2P subunits. Therefore, this novel K 2P subunit was named TREK2 (gene KCNK10 in the human genome organization (HUGO) nomenclature). TREK2
5 shares 63% identity and 78% homology with TREK1. The homology level falls to 69% with TRAAK and to 50-55% with the other K 2P subunits.

II.2. TREK2 gene organization and location.

10 The genomic organization of TREK2 was deduced from the alignment of the cloned cDNAs with the genomic sequences available in the HTGS DNA database. The ORF is composed of six introns and seven exons. The amino-terminus of TREK2 is encoded by exon 1, the M1 domain by exon 2, M2
15 by exon 4, M3 by exon 5 and M4 by exon 6. The third exon codes for the carboxy-terminal part of the M1P1 interdomain and the seventh one encodes the large carboxy-terminus of the channel (Fig. 1A). The length of introns 2-6 varies from 1.8 kb to 35 kb (Fig. 1B). The first exon being out of
20 the genomic contig, the size of the first intron is not known. At this point, it cannot be excluded that the 5' untranslated sequence corresponds to more than one exon.

This organization is different of TWIK1 and TASK3 gene organizations. TWIK1 contains three exons
25 separated by two large introns (23) and TASK3 contains only one short intron (10). However, genomic organization of TREK2 is very close to the genomic organization of both TRAAK (24) and TREK1 channels (unpublished results) genomic organizations. Introns 2 to 6 are found in the same
30 positions. This observation confirms that these three

channels are closely related and suggests that they have arisen by gene duplication from a common ancestor. A particular feature found in TWIK1, TASK1, TREK1, TREK2, TRAAK, TASK2 and TASK3 genes is the presence of a conserved intron in the sequence coding the P1 domain (third intron in the TREK2 gene). The intron site is between the first and the second nucleotides of the codon coding for the first glycine residue of the pore signature sequence G-Y/F/L-G. An intron in the same position is found in 20 genes among the 36 examined that encode potential K⁺ 2P channels in the nematode *Caenorhabditis Elegans* (25) and in 8 genes among 11 in the *Drosophila* as determined by analyzing its recently released genomic sequences. The significance of this conserved intron position is not known, however it is worth noting that this intron has been conserved in mammals where it might eventually have the same role than in the nematode. The analysis of genomic contig bearing the TREK2 gene showed that this sequence contains two Sequence Tag Sites (STS), D14S1058 and WI-6710. WI-6710 has been placed on the WICGR radiation hybrid map 308.53 cR from top of Chr14 linkage group and D14S1058 has been mapped by Genethon 86.3 cM from top of Chr14 linkage group. These results are in agreement and indicate that the chromosomal location of TREK2 gene is 14q31. This location is different from those of TREK1 (1q41) (26) and TRAAK (11q13) (24).

II.3. Tissue distribution of TREK2.

The expression of TREK2 in various adult human tissues was examined by RT-PCR analysis. As shown in Fig.

2A, TREK2 is abundantly expressed in kidney and pancreas, and more moderately in testis, brain, colon and small intestine. Only very faint signals were obtained in liver, heart, prostate and thymus. This expression pattern contrasts with the TREK1 and TRAAK tissue distributions (Fig. 2A). Some tissues express only one of these channels: for instance, pancreas and colon (TREK2), placenta (TRAAK) and ovary (TREK1). Other tissues do not express these channels or only to modest levels: heart, skeletal muscle, lung, PBL and spleen. Finally, some tissues express two or three of these related channels: brain, testis and small intestine. Distributions of TREK1, TREK2 and TRAAK in the different areas of the human brain were analyzed by Northern blot. As shown in Fig. 2B, the TREK2 probe detected two transcripts of 4 and 7.5 kb. TREK2 is mainly expressed in cerebellum, occipital lobe, putamen and thalamus and to lower levels in the other examined areas. No expression was detected in amygdala and spinal cord. The 4 kb transcript is expressed at a higher level than the 7.5 kb transcript except in occipital lobe and cerebellum. As expected from the previous studies on TREK1 and TRAAK expression in rodent central nervous system (2, 6, 20), these two channels have a widespread distribution in the human brain. The 2 kb TRAAK transcript and the 2.7 and 3.3 kb TREK1 transcripts are well expressed in areas where TREK2 is mainly expressed: putamen and thalamus. In the brain cortex (occipital, frontal and temporal lobes), TRAAK is also highly expressed. Finally, TREK1 is the only channel of this family to be expressed in the spinal cord.

II.4. Biophysical properties of TREK2.

TREK2-transfected COS cells display noninactivating currents (Fig. 3A) that are not present in control cells (not shown). The activation kinetics of TREK2 current are rapid. Depolarization pulses induce a two-step current composed of instantaneous and delayed components (Fig. 3A).

The current-voltage (I-V) relationship is outwardly rectifying, and almost no inward currents were recorded in an external medium containing 5 mM K^+ (Fig. 3B). When cells are bathed in a K^+ -rich solution (150 mM), an inward current is revealed and the reversal potential becomes 0 mV as expected for a K^+ -selective channel. However, the I-V relationship is not linear and does not strictly fits the GHK equation for an open K^+ -selective pore. The current has a tendency to saturate at very negative potentials. Two-step activation kinetics and outward rectification in symmetrical K^+ conditions have also been found for the TREK1 current (2, 17). Moreover like TREK1, TREK2 outward currents are more important in 150 mM K^+ than in 5 mM K^+ for depolarizations higher than +50 mV. This effect is unusual since an increase of external K^+ lowers the chemical driving force for outward K^+ flux and would be expected to decrease rather than increase the currents. For TREK1, this effect has been attributed to a stimulating effect of external K^+ as found for other types of K^+ channels (27, 28). In addition, TREK1 has been shown to be sensitive to external Na^+ (Na^+_o). When Na^+_o was substituted by NMDG, TREK1 activity was strongly decreased (2). TREK2 is only partially inhibited by

removing Na^+ . (23% of inhibition, $n = 5$, not shown). Single-channel properties of TREK2 are illustrated in Fig. 3, C and D. Basal channel activity in outside-out patches is characterized by a flickery bursting behaviour (Fig. 3C). In physiological K^+ conditions, the I-V relationship is outwardly rectifying and almost no inward currents were recorded as in whole-cell recording. In symmetrical conditions, inward currents were recorded in addition to outward currents with single-channel conductances of 128 pS at -40 mV and 100pS at +40 mV ($n = 5$) (Fig. 3, C and D). It is interesting to note that the single-channel I-V relationship is inwardly rectifying because the single-channel conductance increases for negative potentials. However, the channel open probability at negative potentials is lower than at positive potentials ($P_o = 0.26$ at -40 mV and $P_o = 0.45$ at +40 mV, $n = 5$) and this explains why the currents are outwardly rectifying in the whole-cell configuration.

II.5. Stretch- and pH-sensitivity of TREK2 currents.

In addition to be modulated by polyunsaturated fatty acids, TREK2 as TREK1 is stimulated by a stretch of the membrane as well as by acidification of the intracellular medium. Fig. 4A shows that application of a negative pressure in the inside-out configuration induces a strong activation of TREK2 activity that is reversible. The activation is graded in function of the applied pressure (Fig. 4B). As shown for TREK1 and TRAAK (17, 18), stretch-induced TREK2 channel activity can be elicited at both

negative and positive potentials and the level of activation increases with depolarization (Fig. 4C). Fig. 4D shows that intracellular acidification induces a strong increase of TREK2 channel activity. This effect is reversible and is observed at all membrane potentials (Fig. 4E). A similar effect has been previously described for TREK1 (19). Acidification of the extracellular medium has no effect on the whole cell TREK2 current (less than 20% of inhibition at pH 6.5) (not shown).

10

II.6. Pharmacological properties.

TREK2 currents are insensitive to tetraethylammonium (TEA, 10 mM) and Ba^{2+} (1 mM). Quinidine inhibited the currents (50% of inhibition at 100 μ M) (not shown). Like TREK1, TREK2 is stimulated by application of the inhalational anesthetics chloroform, halothane and isoflurane (Fig. 5, A and B). At a clinical dose of halothane (29), TREK2 is markedly activated (1.4 ± 0.1 fold increase at 0.25 mM, $n = 10$ at +100 mV). The maximal halothane effect is nearly obtained at 0.5 mM (2.6 ± 0.3 , fold increase, $n = 10$ at +100 mV). The efficiency of anesthetics is different between TREK1 and TREK2. For TREK2, halothane (2.3 ± 0.3 fold increase at 1 mM, $n = 6$ at 0 mV) is more efficient than isoflurane (1.9 ± 0.1 fold increase at 1 mM, $n = 6$) and chloroform (1.8 ± 0.1 fold increase at 1 mM, $n = 7$). For TREK1, chloroform is more effective than halothane and isoflurane at the same concentrations (1 mM) (16). Fig. 5, C and D show that TREK2 is also activated by application of the neuroprotective drug riluzole. As for TREK1, this activation is transient

and is followed by a decrease of the activity corresponding to an inhibition. In the case of TREK1, this is due to an increase of the intracellular cAMP and a phosphorylation of the channel by PKA (30).

5

II.7. Activation of TREK2 by fatty acids and inhibition by intracellular cAMP.

Fig. 6A illustrates the strong stimulating effect of 10 μ M arachidonic acid on TREK2 current (8.4 ± 1.9 fold increase at 0 mV, $n = 6$). This effect is reversible (not shown).

Like TREK1, TREK2 is activated by other polyunsaturated fatty acids, docosahexaenoic and linoleic acids, and by lysophosphatidylcholine, but not by the saturated fatty acid palmitic acid (Fig. 6, B and C) (17, 31). TREK2 is also activated by 10 μ M lysophosphatidylinositol (5.1 ± 0.6 fold increase at 0 mV, $n = 8$). Application of the permeant CPT-cAMP (500 μ M) led to 50% inhibition of TREK2 activity at 0 mV (50 ± 5 , $n = 8$) (Fig. 6D). A similar inhibition is obtained by application of a mixture of 3-isobutyl-1-methylxanthine (IBMX, 1 mM) / forskolin (10 μ M) to increase the intracellular cAMP level (72 ± 10 % of inhibition, $n = 8$). This suggests that TREK2 as TREK1 is inhibited by PKA phosphorylation (16).

25

II.8. Regulation of TREK2 by co-expression with Gs-, Gi- and Gq-coupled neurotransmitter receptors.

TREK2 was co-expressed with 5HT4sR, a Gs-coupled receptor. The stimulation of the receptor by application of 5-HT is associated with a decrease of TREK2

30

activity, as expected for a receptor positively coupled to adenylyate cyclase (Fig. 7A). Conversely, activation of the co-expressed Gi-coupled mGluR2 receptor by glutamate leads to a stimulation of TREK2 activity (Fig. 7B). The decrease
5 of TREK2 activity by the stimulation of 5HT4sR is rapidly reversed after washing (Fig. 7A) while the TREK2 increase associated with mGluR2 is much slower to reverse (more than 10 minutes) (Fig. 7B). A third type of G-protein coupled receptor was co-expressed with TREK2. This receptor is the
10 Gq-coupled mGluR1 receptor. Activation of mGluR1 by application of glutamate led to an inhibition of TREK2 activity that is rapidly reversed by washing (Fig. 7C). The Gq- protein is commonly associated with activation of phospholipase C and the consequent production of
15 diacylglycerol (DAG) and inositol 1,4,5- triphosphate (IP 3). Ultimately, DAG leads to activation of PKC and IP 3 to an increase of intracellular Ca^{2+} . However, neither the application of the PKC-activator PMA (100 nM) nor the addition of Ca^{2+} in the recording pipette (1 μ M) were able
20 to induce an inhibition of TREK2 (not shown).

III. Discussion of the results.

III.1. Characterization of a novel channel.

TREK2 is a novel member of the fatty acid-
25 activated and mechanosensitive K⁺ channel family that includes TREK1 and TRAAK. Like these channels, TREK2 is not blocked by TEA and Ba²⁺ and is stimulated by polyunsaturated fatty acids such as arachidonic, docosahexanoic and linoleic acids, by lysophospholipids and
30 by application of a negative pressure to the cell membrane.

In addition, TREK2 shares with these channels the same gene organization indicating that the three genes probably derive from a common ancestral gene. However, TREK2 is more related to TREK1 than to TRAAK. TREK2 and TREK1 have unique functional and pharmacological properties which are not shared by TRAAK: they are negatively regulated by agents that activate PKA, they are positively regulated by acidification of internal medium and they are strongly activated by volatile general anesthetics. Like TREK1, TREK2 is also transiently activated by riluzole while TRAAK is permanently activated. For TREK1, the inhibition that follows activation by riluzole has been related to an increase of intracellular cAMP and a consequent inhibitory PKA-phosphorylation of the channel (30). In molecular terms, TREK2 is also more related to TREK1, not only if one considers the overall sequence homology but also the distribution of this homology along the sequences. TRAAK, TREK1 and TREK2 have a conserved domain that extends from M1 to M4. Between TREK1 and TREK2, the homology level remains high after M4 and continues over 50 residues. This post-M4 carboxy terminal part is crucial for TREK1 channel sensitivity to fatty acids and stretch but also to PKA and pHi (17, 16, 19). The high level of conservation in this domain between TREK1 and TREK2 explains why these channels have closely related mechanisms of regulation. Interestingly, the PKA site which is implicated in the negative regulation by phosphorylation of TREK1 (17) is conserved in TREK2 (serine 359) suggesting that TREK2 is negatively regulated by PKA in the same way than TREK1. In TREK1, the cytoplasmic amino terminus is not important for

the channel activity and for its mechanical and chemical regulations (17, 16, 19). This is also the case for TREK2 because a truncated TREK2 beginning at methionine 55 apparently conserves its properties after the removal of the first 54 residues by mutagenesis (not shown). The cloning of a novel channel from rat has been published on line (32). This channel is clearly the rat ortholog of human TREK2. These channels have a similar tissue distribution except in the kidney where TREK2 is not expressed in the rat while it is highly expressed in the human. In addition, they share many common functional properties such as single channel conductance and sensitivity to polyunsaturated fatty acids and stretch. However, despite of a high sequence identity (more than 70%), the cytoplasmic amino-terminal part encoded by the first exon (Fig. 1A) is clearly unrelated between these two channels suggesting alternative splicing from a single gene.

III.2. What could be the physiological role of TREK2?

In neurons cultured from mesencephalic and hypothalamic areas of rat brain, several arachidonic acid-activated and mechanosensitive K^+ currents have been characterized (15). Their functional properties are very similar to the properties of TREK-related channels.

Three different native currents have been identified in neurons with I-V relationships being slightly outwardly rectifying or linear or slightly inwardly rectifying, in high symmetrical K^+ conditions. Under the same conditions, the I-V relationship of TREK1 is slightly

outwardly rectifying and the I-V relationship of TRAAK is linear. These results, together with the fact that TREK1 and TRAAK are expressed in brain areas that contain neurons expressing the native currents, suggested that both cloned
5 channels contribute to these native currents. None of the channels cloned until now corresponded to the third type of current with an inward rectification. From the Northern blot analysis, it appears that TREK2 is expressed in the same brain areas as TREK1. Since TREK2 produces currents
10 whose I-V relationship is slightly inwardly rectifying, we propose that TREK2 could form or contribute to the formation of this third type of native arachidonic acid-activated and mechanosensitive current with inward rectification. These channels are expected to play a role
15 in the control of neuronal excitability and particularly, in the control of the resting membrane potential, if they are active at rest in vivo. The level of TREK2 activity can be regulated by the three different types of G-protein coupled receptors. This indicates that TREK2 activity in
20 neurons is probably fine-tuned by a variety of neurotransmitters and that TREK2 could play a role similar to the role of the K^{2P} channel TASK1. In cerebellar granule cells and hypoglossal motoneurons, TASK1 has a central importance in controlling cell excitability and the
25 modulation of its activity by a variety of neurotransmitters acting via Gq-coupled receptors profoundly alters both resting membrane potential and excitability (33, 34). It is interesting to note that the signal transduction pathway by which Gq-coupled receptor
30 inhibits TASK1 does not involve PKC or Ca²⁺ (33, 34) as

also observed for TREK2 . A major difference between TASK1 and TREK2 is that TREK2 is also regulated via Gi- and Gs-coupled receptors. TREK2 will probably turn out to be an important channel in charge of tuning neuronal excitability in response to a variety of neurotransmitters and hormones. The isolation and the characterization of TREK2 constitute an additional step toward the understanding of this particular class of K + channels which probably plays a wide variety of important physiological roles in the brain and other tissues (11) and which, because it is a target of volatile anesthetics ((16) and this article) and riluzole, a neuroprotective drug (6, 30), might have an important impact in medicine.

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CLAIMS

1) A protein constituting a mammalian K⁺ channel
with two pore domains that produces currents whose current-
5 voltage relationship is slightly inwardly rectifying in
high symmetrical K⁺ conditions.

2) A protein according to claim 1 wherein said
mammalian K⁺ channel is a human K⁺ channel.

10

3) A protein according to any of claims 1 or 2,
wherein said protein is constituted by or comprises the
sequence of amino acids in the list of sequences under the
number SEQ ID No. 2.

15

4) A nucleic acid molecule comprising or
constituted of an encoding nucleic sequence for a mammalian
K⁺ channel with two pore domains that produces currents
whose current-voltage relationship is slightly inwardly
20 rectifying in high symmetrical K⁺ conditions or for a
fragment of such a mammalian K⁺ channel.

5) A nucleic acid molecule according to claim
4, wherein said mammalian K⁺ channel is a human K⁺ channel.

25

6) A nucleic acid molecule according to any of
claims 4 or 5, whose amino acid sequence is represented in
the list of sequences in the appendix under the number SEQ
ID No. 2.

7) A nucleic acid molecule according to any of claims 4 to 6, constituted by or comprising the sequence in the list of sequences in the appendix under the number SEQ ID No. 1.

5

8) A polyclonal or monoclonal antibody directed against a mammalian K⁺ channel according to any of claims 1 to 3 a derivative or a fragment of said antibody.

10

9) A vector comprising at least one molecule of nucleic acid according to any of claims 4 to 7, advantageously associated with adapted control sequences.

15

10) A cellular host transformed by one molecule of nucleic acid according to any of claims 4 to 7.

11) A cellular host transformed by a vector according to claim 9.

20

12) A nucleic and oligonucleotide probe prepared from one molecule of nucleic acid according to any of claims 4 to 7.

25

13) A method for identifying a biologically active compound with anesthetics properties comprising:

30

(a) contacting said compound with a cellular host expressing on its surface a mammalian K⁺ channel with two pore domains whose current-voltage relationship is slightly inwardly rectifying in high symmetrical K⁺ conditions,

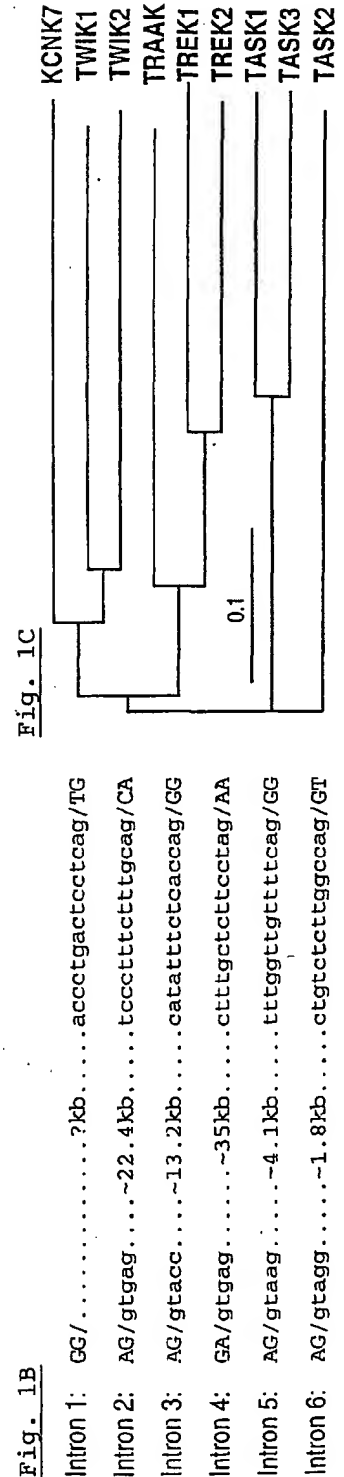
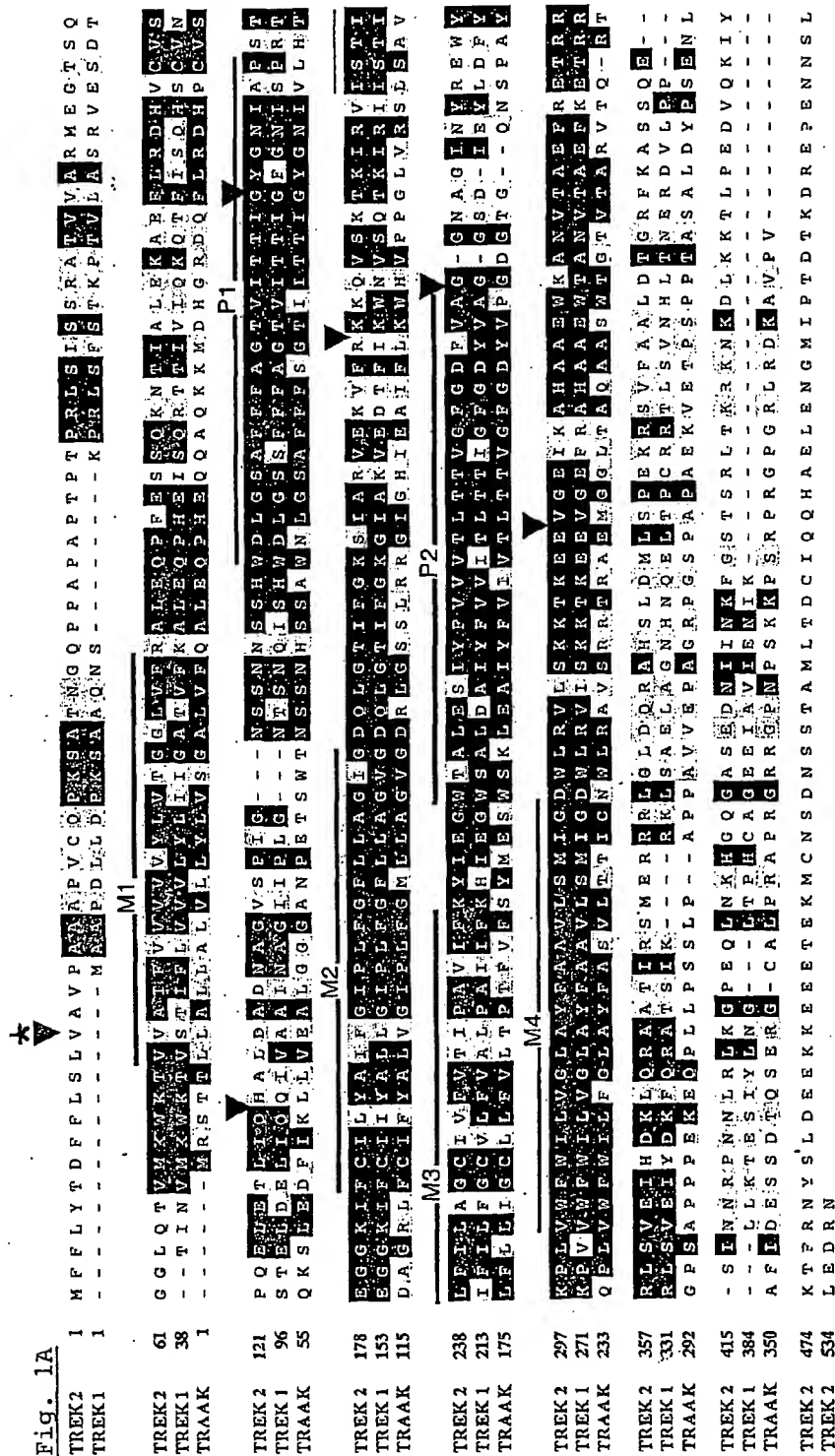
(b) determining the K^+ transport activity of said mammalian K^+ channel wherein an activation of K^+ transport is indicative of said compound having anesthetics properties.

5

14) A method according to claim 13, wherein said mammalian K^+ channel is a human K^+ channel.

10 15) A method according to any of claims 13 or 14, wherein said mammalian K^+ channel is constituted by or comprising the sequence of amino acids in the list of sequences under the number SEQ ID No. 2.

15 16) A pharmaceutical composition containing a biologically active compound with anesthetics properties identified by a method according to any of claims 13 to 15.



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Fig. 2A

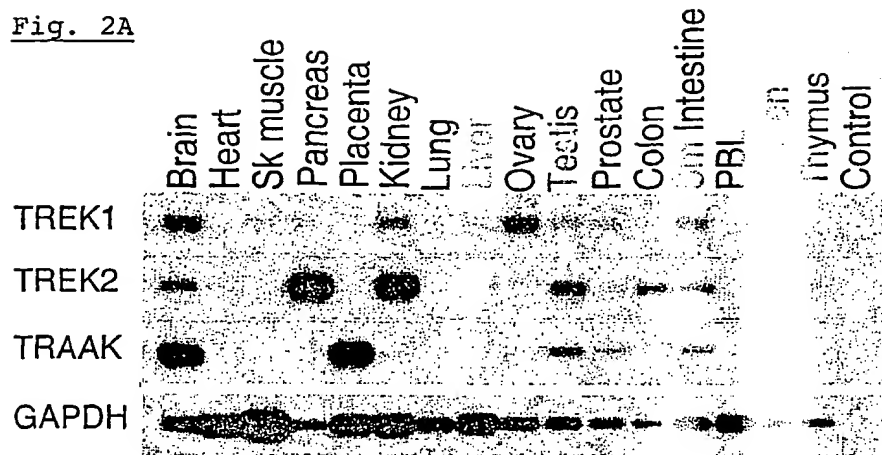
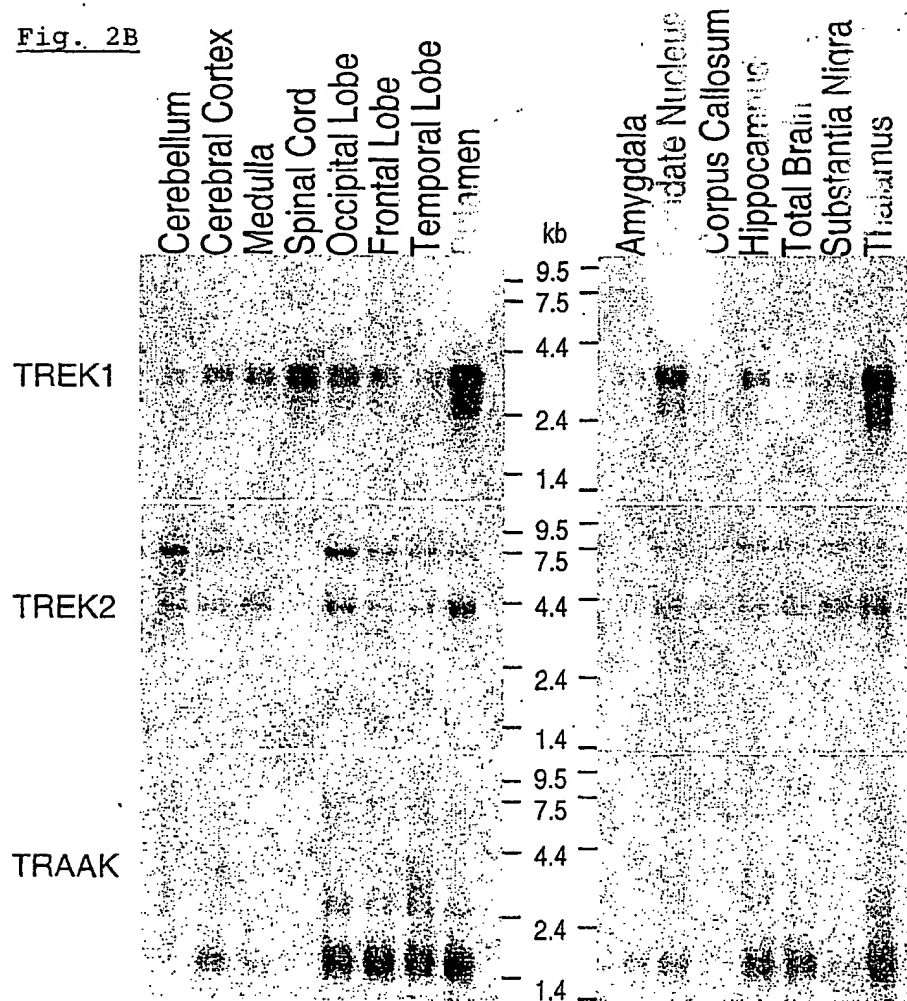


Fig. 2B



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Fig. 3A

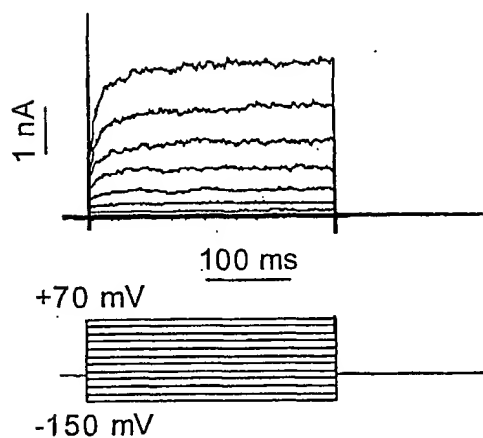


Fig. 3B

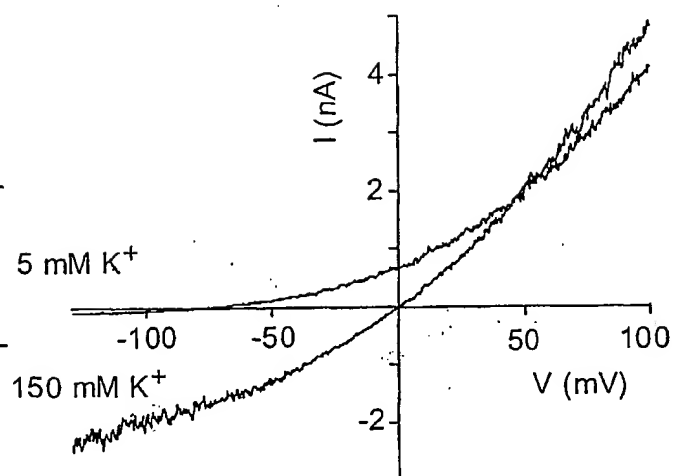


Fig. 3C

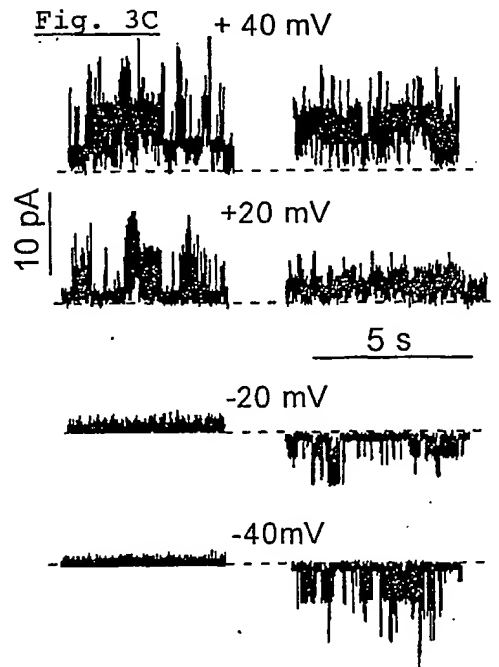
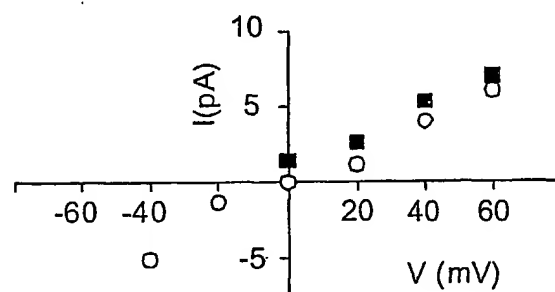


Fig. 3D



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Fig. 4A

+50 mV



Fig. 4B

0 mV

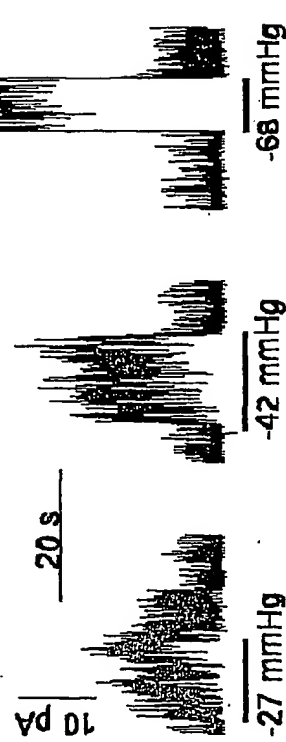


Fig. 4C

-40 mV

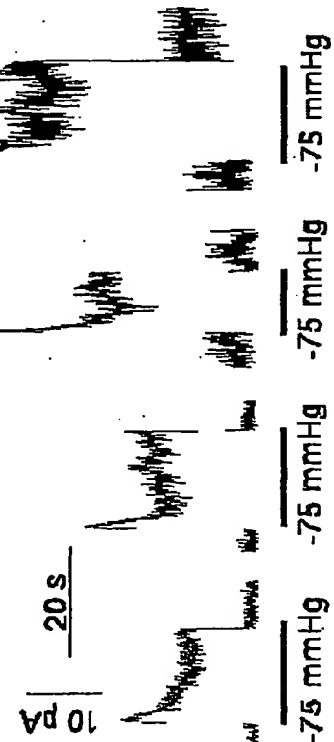
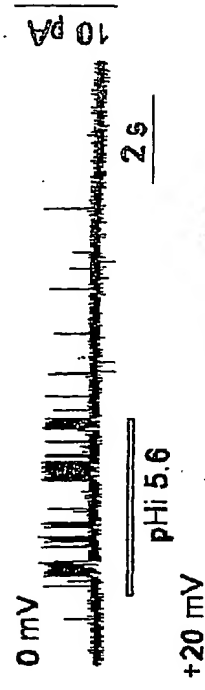
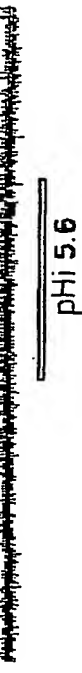


Fig. 4D



+20 mV



+40 mV

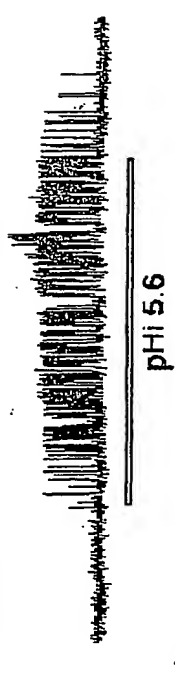
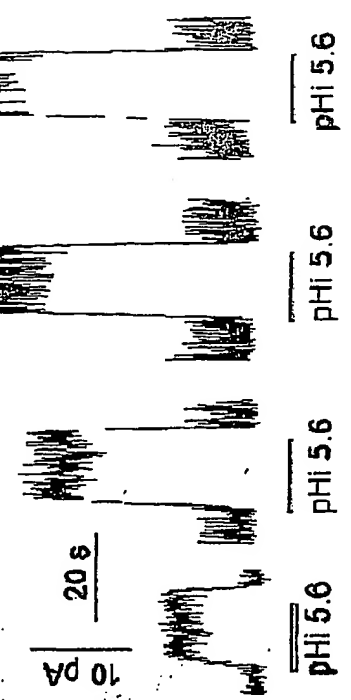
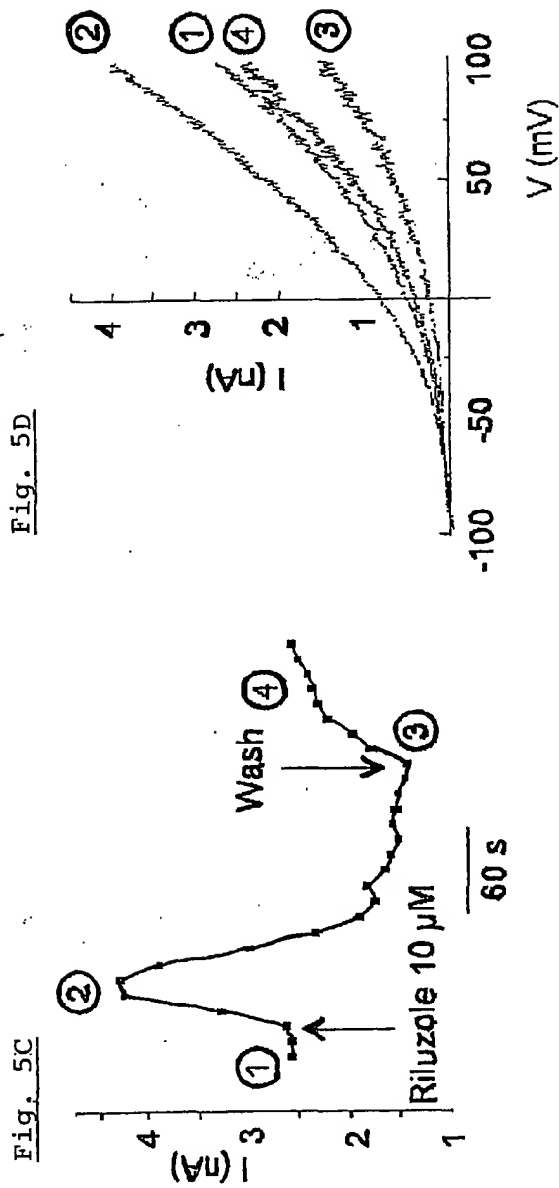
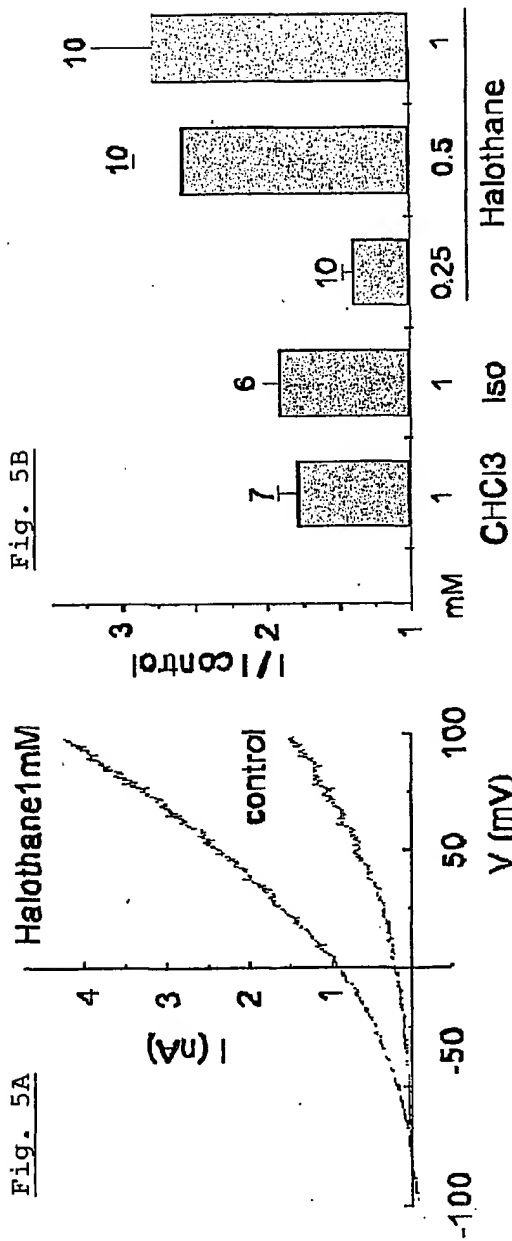


Fig. 4E

-40 mV





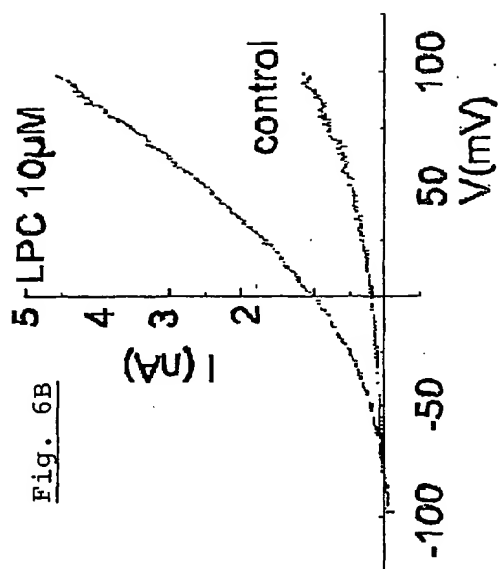


Fig. 6B

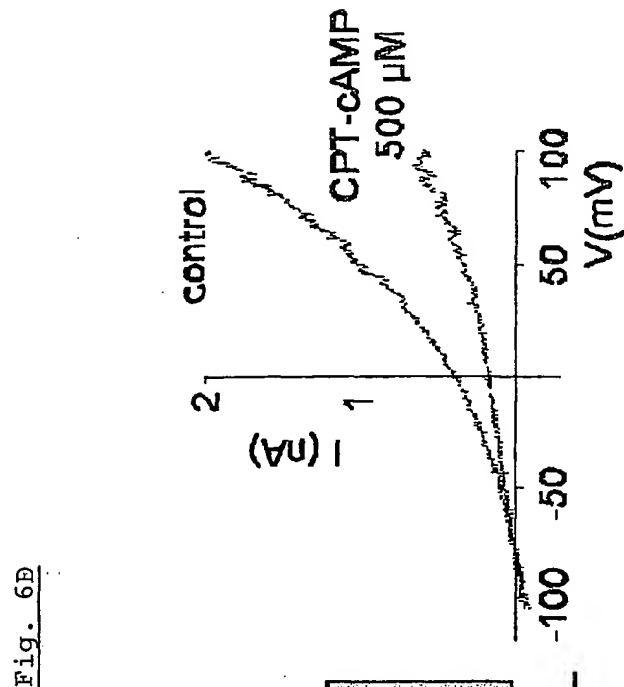
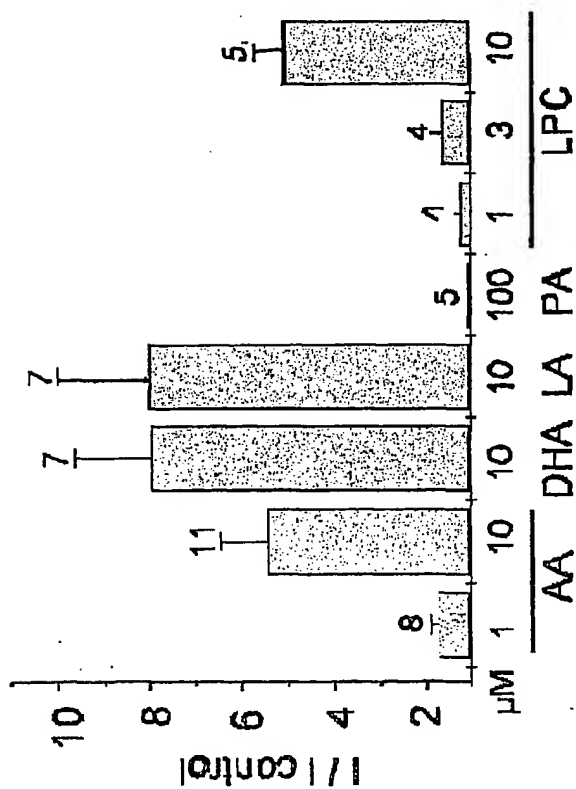
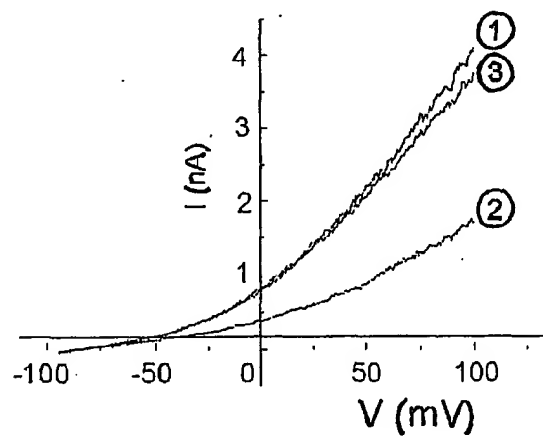
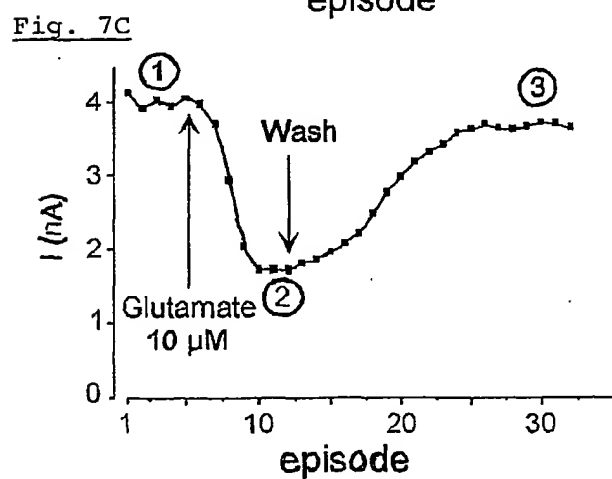
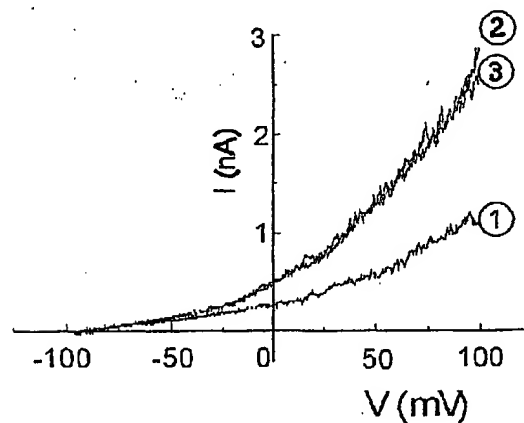
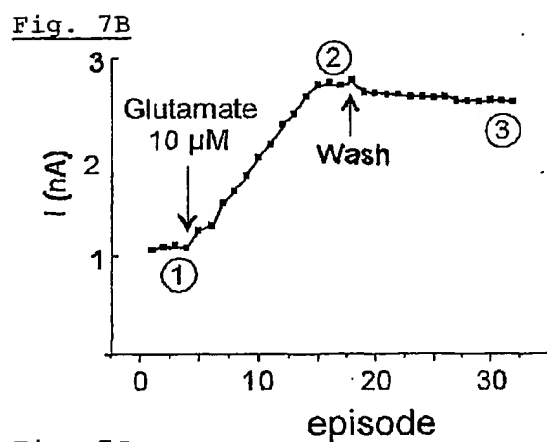
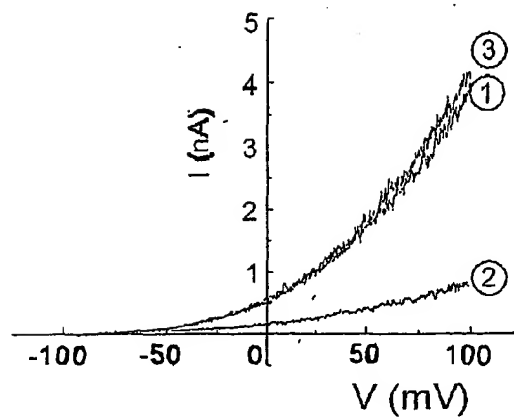
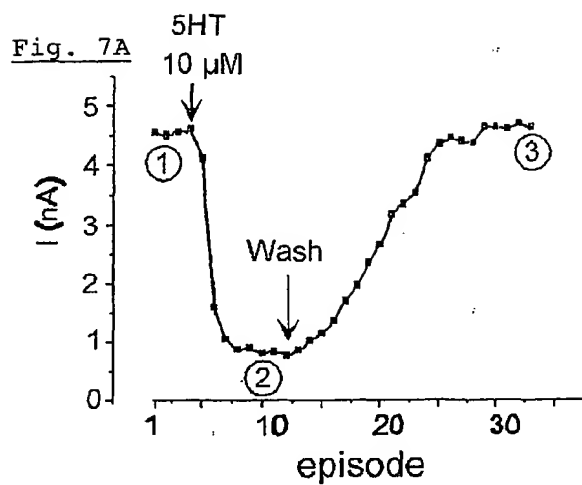


Fig. 6D



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SEQUENCE LISTING

<110> Centre National de la Recherche Scientifique

<120> Human TREK2, a novel stretch- and arachidonic acid-sensitive K⁺ channel activated by inhalational anesthetics and riluzole

<130> 8606PCT

<140> 8606PCT

<141> 2001-06-27

<150> US 60/214.559

<151> 2000-06-27

<160> 17

<170> PatentIn version 3.0

<210> 1

<211> 1614

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (1)..(1614)

<223> ORF of human TREK2 cDNA

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gca	gca	gca	ccg	gtg	tgc	cag	ccc	aag	agc	gcc	act	aac	ggg	caa	ccc	96
Ala	Ala	Ala	Pro	Val	Cys	Gln	Pro	Lys	Ser	Ala	Thr	Asn	Gly	Gln	Pro	
			20					25					30			

ccg	gct	ccg	gct	ccg	act	cca	act	ccg	cgc	ctg	tcc	att	tcc	tcc	cga	144
Pro	Ala	Pro	Ala	Pro	Thr	Pro	Thr	Pro	Arg	Leu	Ser	Ile	Ser	Ser	Arg	
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gcc	aca	gtg	gta	gcc	agg	atg	gaa	ggc	acc	tcc	caa	ggg	ggc	ttg	cag	192
Ala	Thr	Val	Val	Ala	Arg	Met	Glu	Gly	Thr	Ser	Gln	Gly	Gly	Leu	Gln	
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acc	gtc	atg	aag	tgg	aag	acg	gtg	gtt	gcc	atc	ttt	gtg	gtt	gtg	gtg	240
Thr	Val	Met	Lys	Trp	Lys	Thr	Val	Val	Ala	Ile	Phe	Val	Val	Val	Val	
65					70				75					80		

gtc	tac	ctt	gtc	act	ggc	ggt	ctt	gtc	ttc	cgg	gca	ttg	gag	cag	ccc	288
Val	Tyr	Leu	Val	Thr	Gly	Gly	Leu	Val	Phe	Arg	Ala	Leu	Glu	Gln	Pro	
			85					90					95			

ttt	gag	agc	agc	cag	aag	aat	acc	atc	gcc	ttg	gag	aag	gcg	gaa	ttc	336
Phe	Glu	Ser	Ser	Gln	Lys	Asn	Thr	Ile	Ala	Leu	Glu	Lys	Ala	Glu	Phe	

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ctg cgg gat cat gtc tgt gtg agc ccc cag gag ctg gag acg ttg atc			384
Leu Arg Asp His Val Cys Val Ser Pro Gln Glu Leu Glu Thr Leu Ile			
115	120	125	
cag cat gct ctt gat gct gac aat gcg gga gtc agt cca ata gga aac			432
Gln His Ala Leu Asp Ala Asp Asn Ala Gly Val Ser Pro Ile Gly Asn			
130	135	140	
tct tcc aac aac agc agc cac tgg gac ctc ggc agt gcc ttt ttc ttt			480
Ser Ser Asn Asn Ser Ser His Trp Asp Leu Gly Ser Ala Phe Phe Phe			
145	150	155	160
gct gga act gtc att acg acc ata ggg tat ggg aat att gct ccg agc			528
Ala Gly Thr Val Ile Thr Thr Ile Gly Tyr Gly Asn Ile Ala Pro Ser			
165	170	175	
act gaa gga ggc aaa atc ttt tgt att tta tat gcc atc ttt gga att			576
Thr Glu Gly Gly Lys Ile Phe Cys Ile Leu Tyr Ala Ile Phe Gly Ile			
180	185	190	
cca ctc ttt ggt ttc tta ttg gct gga att gga gac caa ctt gga acc			624
Pro Leu Phe Gly Phe Leu Leu Ala Gly Ile Gly Asp Gln Leu Gly Thr			
195	200	205	
atc ttt ggg aaa agc att gca aga gtg gag aag gtc ttt cga aaa aag			672
Ile Phe Gly Lys Ser Ile Ala Arg Val Glu Lys Val Phe Arg Lys Lys			
210	215	220	
caa gtg agt cag acc aag atc cgg gtc atc tca acc atc ctg ttc atc			720
Gln Val Ser Gln Thr Lys Ile Arg Val Ile Ser Thr Ile Leu Phe Ile			
225	230	235	240
ttg gcc ggc tgc att gtg ttt gtg acg atc cct gct gtc atc ttt aag			768
Leu Ala Gly Cys Ile Val Phe Val Thr Ile Pro Ala Val Ile Phe Lys			
245	250	255	
tac atc gag ggc tgg acg gcc ttg gag tcc att tac ttt gtg gtg gtc			816
Tyr Ile Glu Gly Trp Thr Ala Leu Glu Ser Ile Tyr Phe Val Val Val			
260	265	270	
act ctg acc acg gtg ggc ttt ggt gat ttt gtg gca ggg gga aac gct			864
Thr Leu Thr Thr Val Gly Phe Gly Asp Phe Val Ala Gly Gly Asn Ala			
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Leu Val Gly Leu Ala Tyr Phe Ala Ala Val Leu Ser Met Ile Gly Asp			
305	310	315	320

tgg cta cgg gtt ctg tcc aaa aag aca aaa gaa gag gtg ggt gaa atc	1008
Trp Leu Arg Val Leu Ser Lys Lys Thr Lys Glu Glu Val Gly Glu Ile	
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Lys Ala His Ala Ala Glu Trp Lys Ala Asn Val Thr Ala Glu Phe Arg	
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Glu Thr Arg Arg Arg Leu Ser Val Glu Ile His Asp Lys Leu Gln Arg	
355 360 365	
gcg gcc acc atc cgc agc atg gag cgc cgg cgg ctg ggc ctg gac cag	1152
Ala Ala Thr Ile Arg Ser Met Glu Arg Arg Arg Leu Gly Leu Asp Gln	
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Arg Ala His Ser Leu Asp Met Leu Ser Pro Glu Lys Arg Ser Val Phe	
385 390 395 400	
gct gcc ctg gac acc ggc cgc ttc aag gcc tca tcc cag gag agc atc	1248
Ala Ala Leu Asp Thr Gly Arg Phe Lys Ala Ser Ser Gln Glu Ser Ile	
405 410 415	
aac aac cgg ccc aac aac ctg cgc ctg aag ggg ccg gag cag ctg aac	1296
Asn Asn Arg Pro Asn Asn Leu Arg Leu Lys Gly Pro Glu Gln Leu Asn	
420 425 430	
aag cat ggg cag ggt gcg tcc gag gac aac atc atc aac aag ttc ggg	1344
Lys His Gly Gln Gly Ala Ser Glu Asp Asn Ile Ile Asn Lys Phe Gly	
435 440 445	
tcc acc tcc aga ctc acc aag agg aaa aac aag gac ctc aaa aag acc	1392
Ser Thr Ser Arg Leu Thr Lys Arg Lys Asn Lys Asp Leu Lys Lys Thr	
450 455 460	
ttg ccc gag gac gtt cag aaa atc tac aag acc ttc cgg aat tac tcc	1440
Leu Pro Glu Asp Val Gln Lys Ile Tyr Lys Thr Phe Arg Asn Tyr Ser	
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ctg gac gag gag aag aaa gag gag gag acg gaa aag atg tgt aac tca	1488
Leu Asp Glu Glu Lys Lys Glu Glu Glu Thr Glu Lys Met Cys Asn Ser	
485 490 495	
gac aac tcc agc aca gcc atg ctg acg gac tgt atc cag cag cac gct	1536
Asp Asn Ser Ser Thr Ala Met Leu Thr Asp Cys Ile Gln Gln His Ala	
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gag ttg gag aac gga atg ata ccc acg gac acc aaa gag cgg gag ccg	1584
Glu Leu Glu Asn Gly Met Ile Pro Thr Asp Thr Lys Asp Arg Glu Pro	
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gag aac aac tca tta ctt gaa gac aga aac	1614
Glu Asn Asn Ser Leu Leu Glu Asp Arg Asn	

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Pro Ala Pro Ala Pro Thr Pro Thr Pro Arg Leu Ser Ile Ser Ser Arg
 35 40 45

Ala Thr Val Val Ala Arg Met Glu Gly Thr Ser Gln Gly Gly Leu Gln
 50 55 60

Thr Val Met Lys Trp Lys Thr Val Val Ala Ile Phe Val Val Val Val
 65 70 75 80

Val Tyr Leu Val Thr Gly Gly Leu Val Phe Arg Ala Leu Glu Gln Pro
 85 90 95

Phe Glu Ser Ser Gln Lys Asn Thr Ile Ala Leu Glu Lys Ala Glu Phe
 100 105 110

Leu Arg Asp His Val Cys Val Ser Pro Gln Glu Leu Glu Thr Leu Ile
 115 120 125

Gln His Ala Leu Asp Ala Asp Asn Ala Gly Val Ser Pro Ile Gly Asn
 130 135 140

Ser Ser Asn Asn Ser Ser His Trp Asp Leu Gly Ser Ala Phe Phe Phe
 145 150 155 160

Ala Gly Thr Val Ile Thr Thr Ile Gly Tyr Gly Asn Ile Ala Pro Ser
 165 170 175

Thr Glu Gly Gly Lys Ile Phe Cys Ile Leu Tyr Ala Ile Phe Gly Ile

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Pro Leu Phe Gly Phe Leu Leu Ala Gly Ile Gly Asp Gln Leu Gly Thr		
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Ile Phe Gly Lys Ser Ile Ala Arg Val Glu Lys Val Phe Arg Lys Lys		
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Gln Val Ser Gln Thr Lys Ile Arg Val Ile Ser Thr Ile Leu Phe Ile		
225	230	235
Leu Ala Gly Cys Ile Val Phe Val Thr Ile Pro Ala Val Ile Phe Lys		
245	250	255
Tyr Ile Glu Gly Trp Thr Ala Leu Glu Ser Ile Tyr Phe Val Val Val		
260	265	270
Thr Leu Thr Thr Val Gly Phe Gly Asp Phe Val Ala Gly Gly Asn Ala		
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Gly Ile Asn Tyr Arg Glu Trp Tyr Lys Pro Leu Val Trp Phe Trp Ile		
290	295	300
Leu Val Gly Leu Ala Tyr Phe Ala Ala Val Leu Ser Met Ile Gly Asp		
305	310	315
Trp Leu Arg Val Leu Ser Lys Lys Thr Lys Glu Glu Val Gly Glu Ile		
325	330	335
Lys Ala His Ala Ala Glu Trp Lys Ala Asn Val Thr Ala Glu Phe Arg		
340	345	350
Glu Thr Arg Arg Arg Leu Ser Val Glu Ile His Asp Lys Leu Gln Arg		
355	360	365
Ala Ala Thr Ile Arg Ser Met Glu Arg Arg Arg Leu Gly Leu Asp Gln		
370	375	380
Arg Ala His Ser Leu Asp Met Leu Ser Pro Glu Lys Arg Ser Val Phe		
385	390	395
		400

Ala Ala Leu Asp Thr Gly Arg Phe Lys Ala Ser Ser Gln Glu Ser Ile
 405 410 415

Asn Asn Arg Pro Asn Asn Leu Arg Leu Lys Gly Pro Glu Gln Leu Asn
 420 425 430

Lys His Gly Gln Gly Ala Ser Glu Asp Asn Ile Ile Asn Lys Phe Gly
 435 440 445

Ser Thr Ser Arg Leu Thr Lys Arg Lys Asn Lys Asp Leu Lys Lys Thr
 450 455 460

Leu Pro Glu Asp Val Gln Lys Ile Tyr Lys Thr Phe Arg Asn Tyr Ser
 465 470 475 480

Leu Asp Glu Glu Lys Lys Glu Glu Glu Thr Glu Lys Met Cys Asn Ser
 485 490 495

Asp Asn Ser Ser Thr Ala Met Leu Thr Asp Cys Ile Gln Gln His Ala
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<212> DNA
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<222> (1)..(22)
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22

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20

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<222> (1)..(20)
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<400> 16
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<210> 17
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<222> (1)..(25)
<223> 32P-labeled primer (TREK1)
<400> 17
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